Szent István University
Doctorate Course of Veterinary Science

The study of some ecotoxicological properties of doxycycline used for mass treatment of production animals

PhD thesis

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Introduction, main objectives

The basic condition of the application of a veterinary pharmaceutical is the documentation of the proven existence of its appropriate efficacy and innocuity. The harmlessness traditionally has meant the safety of the treated (target) animal, as well as of the person treating the animals and the safety of consumer of meat and products of animal origin. Recently, the requirement has been supplemented with the study of the effect of the active substance on the environment and its fauna. This is specifically important in case of veterinary medicinal products used for mass treatment, such as tetracyclines.

The environmental protection is getting a more complex issue today. Besides the refuse disposal, the recycling, total elimination and the rehabilitation of the environment have gained priority. In our life 100 000 chemicals are used, which may endanger the environment by exerting potential toxic effect on plants, animals, humans and on the whole ecosystem. Therefore, overall measures have been taken in most developed countries to control the wide range of environmental pollutants.

The obligation to prepare environmental toxicological assessment in the registration documentation of veterinary pharmaceuticals was prescribed by the Directive 92/18/EC. According to guidelines, the ecotoxicological assessment must be executed in two phases. In the first phase, the potential concentration of the active substance in the environment is calculated, and in the second phase the fate and potential effects of the active substance is evaluated

The purpose is to reduce the environmental risk while using veterinary pharmaceuticals to a minimum level or, if possible, to eliminate it completely. The type and measure of potential risk should be determined. In this framework, the rate of degradation, the kinetics of the active substance (its fate in different matrices: manure, sludge, sediment, soil, water, etc.) must be followed altogether with the determination of potential effects on plants, animals and other living creatures in soil and water, which are representing a given part of the ecosystem.

Among veterinary medicines the tetracycline group is the most important. The members of the group represent different phisico-chemical properties and biological activities. Oxytetracycline, chlortetracycline and tetracycline are belonging to the “traditional” tetracycline group while doxycycline and minocycline are newer derivative. The traditional ones have been used for a long time in veterinary practice and environmental toxicological data are available mainly for these compounds. Recently, doxycycline has been widely used also for mass treatment of production animals.

Compared to traditional tetracyclines, doxycycline is more lipophylic, it is more readily absorbed at oral administration and it has better antimicrobial efficacy. In contrast, information on its fate in the environment, on its degradation rate in manure, in soil, and the impact on soil microflora are hardly available.

In this dissertation the results of experiments on the presence and degradation of doxycycline in manure is described under different conditions. The purpose is to predict its potential presence in the manure used for treatment of arable lands, if the given animal stock is treated with doxycycline. Then, the degradation of the antibiotic in soil was examined by following the fate of doxycycline in manure used for fertilization. In further experiments the aim was to determine the potential effect on the activity of soil mirco-organisms.

Material and method

The degradation rate of doxycycline was determined in two experiments. In both cases the pigs were treated with veterinary medicinal product containing doxycycline and the manure was collected during the treatment period. In the *in vitro* study the manure was kept during the 16-week ageing period in closed 300-ml bottles (usually used for determination of biological oxygen demand) to maintain 20±3,5 °C temperature, humidity and anaerobe conditions according to relevant guidelines (CVMP, 2008, CVMP, 2011). The first sample was taken at the end of the treatment period and then further samples were taken 1, 2, 4, 8, 12 and 16 weeks after the end of the treatment.

In the other study the manure was aged on the field in the Model Farm of the Szent István University, Faculty of Veterinary Science (Üllő). The manure, according to the farm protocol, was exposed to the weather and the environment. Just at finishing the treatment and 1, 2, 3, 6, 8, 10 and 12 weeks after treatment samples were taken. The samples were stored at -30 °C before the processing in both studies.

The concentration of doxycycline in manure samples was determined in the Food-Toxicological Laboratory of the Department of Food Hygiene, Szent István University, Faculty of Veterinary Science. Earlier, a method was developed (Food Toxicological Laboratory of the Department of Food Hygiene, Szent István University Faculty of Veterinary Science) on regulatory experimental purpose for the determination of doxycycline in different biological matrices (blood plasma, liver, kidney, skin, fat, faeces, etc.). This method was adapted and validated for the quantification of doxycycline in pig manure samples. Following sample preparation, doxycycline was assessed by reverse-phase high performance liquid chromatography and UV (diode-array) detection. The method was validated for specificity, linearity, recovery, repeatability and accuracy, limit of detection (LOD) and limit of quantification (LOQ) and stability according to the guidelines of European Medicines Agency.

The soil degradation study was performed as a continuation of the previous field study. The manure composted for 12 weeks was spread as fertilizer on agricultural land utilized for maize cultivation and ploughed (0-45 cm depth) into the soil. Before land application of the doxycycline-laced manure, control samples of the soil were obtained. Then, samples were taken from the field immediately after fertilization and ploughing, and 2, 4, 8, 14 and 20 weeks after manure application. Samples were collected from three different levels of the soil (0 cm, 25 cm and 50 cm depths).

Soil samples were analyzed for doxycycline using solid phase extraction (SPE) followed by high-performance liquid chromatography (HPLC). The applied method developed for detection of tetracycline in soil and described in the literature was adapted and validated to detection of doxycyclin in soil samples. The method was validated for specificity, linearity, recovery, limit of detection (LOD) and limit of quantification (LOQ).

Beyond the degradation of doxycycline under different environmental conditions, the potential effect on soil microorganisms was also examined. The effect of doxycycline on soil microbial activity was measured by the nitrogen transformation test. The nitrogen transformation test was carried out according to the OECD Guideline 216. The soil was originated from the same area used in the soil degradation study. Doxycycline, dissolved in water, was added and mixed thoroughly in 5 different concentrations to the soil (25, 50, 100, 200, 400 μg/kg). Each soil samples was supplemented by lucerne meal as an organic substrate for microorganisms. The incubation was performed at ambient temperature (20±2°C) in the dark for 28 days. The moisture content of the soil was maintained during the whole experiment with added distilled water. The soil samples were taken before treating with the antibiotic, and on day 7, day 14, day 28 of the incubation period. Each sample was extracted with 0.1M KCl solution and the extracts were stored at -20°C before being analyzed for nitrate content. The actual determination of nitrate in the soil extract was performed in accordance with the ISO/TS 14256—1 guidance. First, the nitrate was reduced to nitrite by passing the extract through a reduction column, containing copperized cadmium. Then, nitrite formed a diazo compound in acid medium after addition of sulfanilamide and N-(1-naphthyl) ethylendiamine dihydrochloride (Griess—Ilosvay reagent). Finally, the concentration of nitrite and consequently nitrate was determined spectrophotometrically at 543 nm.

In a further experiment the effect of doxycycline and two other antibiotics on the activity of soil micro flora was studied by measuring the change of redox potential. In the study five different soils and doxycycline, enrofloxacin and lincomycin, respectively were used and compared. Concerning the soil used in the previous experiments, the four other soils had different properties, for example in sand content, pH, organic carbon content. The offered soil types of OECD guide 106 were also taken into consideration. Each antibiotics was solved in distilled water was added to soil samples. Four concentrations and a control soil were used: 200, 400, 800 and 1600 μg/kg. The solution containing the antibiotics was supplemented to 25 ml with distilled water and added to 10 g soil. One ml samples were taken from the soil solution and then were added to 9 ml liquid broth for measuring the possible change of redox potential.

The soil in the experiment served as the source of undefined microbes which were able to grow on standard nutrient media. The basis of the detection method is that bacteria can change the redox potential of the environment by multiplication and/or by change in metabolic rate due to energy producing biochemical reactions and consequently, the redox potential (and correspondingly the TTD value) is decreased. The detection time (TTD=time to detection) is that moment when the absolute value of the rate of redox potential change in the measuring-cell overcomes a value which is significantly differing from the random changes (e.g. $|dE/dt| \geq 1$ mV/min). This value is the detection criterion. The multiplication and activity of microorganisms are detected with MicroTester instrument by measuring the redox potential change of the environment (broth). The detection of the change in the measured value indicates the change of the number of viable cells and/or the rate of metabolism (altogether: activity) of the microorganisms.

Results

In the laboratory study the initial concentration of doxycycline was 61.6 mg/kg. During composting the concentration continuously decreased: after 4 and 8 weeks, 54% and 42% of the initial doxycycline concentration could be detected, respectively (37.7 mg/kg and 32,9 mg/kg). By the end of the 16-week storage period, the initial amount of doxycycline was degraded by up to 70% but even at this time the concentration of doxycycline was still high (20.4 mg/kg) so after four and a half months *in vitro* composting of pig manure the concentration of doxycycline was more than 20 mg/kg. The calculated half-life for doxycycline in manure was 52.5 days (7.5 weeks) in the laboratory study.

In the field study the initial concentration of doxycycline (87.4 mg/kg manure) was higher than in the *in vitro* experiment. The degradation during the 12-week ageing period under environmental conditions proved to be faster than in the laboratory experiment. The originally high concentration of doxycycline already decreased significantly for the first week and only the half of the initial concentration could be detected on the 2nd week (40.00 mg/kg). By the 4th and 8th week of storage, 38.6% and 16.8% of the original concentration of doxycycline could only be detected, respectively compared with the results of *in vitro* study where after 4 and 8 weeks, 54% and 42% of the original doxycycline concentration still could be detected. At the end of the whole storage period of 12 weeks, 89.3% of the initial doxycycline concentration was degraded (to concentration of 9.37 mg/kg). The calculated half-life for doxycycline in manure was 25.7 days (3.68 weeks) in the field study. The results indicate that the degradation in the field study under natural conditions was nearly two times faster than in the laboratory study under controlled conditions.

In the degradation study, the initial concentrations of doxycycline in the soil samples were 0.25 ± 0.03 mg/kg in the surface samples of the agricultural land, 0.188 ± 0.02 mg/kg in the 20-25-cm depth samples and 0.168 ± 0.02 mg/kg in the 45-50-cm depth layers. At the end of the whole manure composting period the concentration of doxycycline in manure was 9.37 mg/kg, so 2-2.5% of the quantity measured in the manure could be detected in soil. After four and eight weeks, 59.7 % and 43.8 % of the initial doxycycline concentration could be measured in the surface soil samples (0.15 mg/kg and 0.11 mg/kg), whereas 75.5 % (0.14 mg/kg) and 57.3 % (0.1 mg/kg) of the initial doxycycline concentration were still detectable in the 20-25-cm and the 45-50-cm depth soil layer samples. The concentration was decreased to 33.3 % (0.06 mg/kg) in 20-25 cm depth only after 14 weeks. By the end of the 20 weeks long sampling period, doxycycline was degraded by 76.2 % on the surface, by 67 % in the 25 cm depth and by 81.2 % in the 50 cm depth soil layers with the following concentrations: 0.06 mg/kg, 0.06 mg/kg and 0.03 mg/kg, respectively. The calculated half-life for doxycycline in the study was 66.5 days (9.5 weeks), 76.3 days (10.9 weeks), 59.4 days (8.5 weeks) at depths of 0 cm, 25 cm and 50 cm, respectively.

In the nitrogen transformation test, the nitrate content was similar at the beginning of the study for each treatment and replicate, ranging from 10 to 15 mg/kg soil. On day 7 an increase in the quantity of nitrate could be seen in each soil sample and this increase in soil samples treated with different quantities of doxycycline remained far below that of the control (60.0 %, 42.9 %, 32.9 %, 42.7 % and 50.0 % nitrate concentration of the control, respectively). The overall increase in the content of nitrate was due to the addition of lucerne meal as an organic substrate and its utilization by soil microorganisms. After 2 weeks of incubation 79.3–88.8 %, on day 28 (last day of the experiment) 76.9 %, 53.0 %, 65.6 %, 59.7 % and 77.1 % of the control nitrate concentrations were measured in the soil treated with different amounts of doxycycline, corresponding to the concentration range of 25–37 mg/kg soil. The difference between concentrations of nitrate as a function of the concentration of doxycycline was significant. Biologically significant difference (>25%) were found in all samples taken at 7th day and in samples taken at 28th day at doxycycline concentrations of 50, 100, 200 μg/kg,

The aim of the application of redox potential test was to study the possible effect of doxycycline on the metabolic activity of soil microorganisms. Doxycycline exhibited effect on redox potential change in all soil types. The TTD value in doxycycline treated relative to control samples showed a 153,5-374,1% increase (indicating inhibition of microbial activity). The slightest effect was in the volcanic ash soil (53,5-84,9% increase in TTD). The effect appeared already at 200 μg/kg and was increased by higher doxycycline concentrations. The effect of enrofloxacin was moderate compared with doxycycline, moreover in case of silt loam soil and loam soil no effect could be found and in silty clay loam soil the effect was limited. In sand soil and volcanic ash soil the effect was similar (51,7-114,3%, and 54,4-121,8% TTD increase). In the latter two soil types at the concentration of 200 μg/kg, the detection time change was significant. The difference in the effects with different soil types also could be observed in case of lincomycin. The strongest effect was in sand soil, silt loam soil and volcanic ash soil (60,0-115,7%, 39,7-85,2% and 100,0-175,8% TTD increase). The effect could be detected even at the concentration of 200 μg/kg.

Conclusion

The degradation rate found in the present field study was faster than that observed in the laboratory study. Possible differences in degradation rate can be partly due to the photodegradation of doxycycline in the manure heap as it was described in other publications, although it was not confirmed in the soil degradation study. On the other hand, the difference can also be explained by other environmental conditions, e.g. the effect of rain, which could rinse the antibiotic from the manure into the soil. Similar differences were found in the degradation of other tetracyclines as well. About 50% of tetracycline was degraded within 4.5 days in ventilated pig manure, while in non-ventilated manure twice as long composting time was needed to reach the same concentration. In the present laboratory trial and in the field study, the degradation half-lives of doxycycline in manure were calculated to be 52.5 days (7.5 weeks) and 25.7 days (3.68 weeks), respectively. Although the bibliographic results apply to tetracycline, oxytetracycline and chlortetracycline, the results obtained from the present studies are in agreement with the data cited above. The results of our studies indicate that the manure originated from herd treated by doxycycline in terapeutic duration and dose, after 3 months composting contained the antibiotic in a concentration of 9-13 mg/kg.

By the end of the 20 weeks long sampling period, thie initial amount of doxycycline (0,25 mg/kg) was degraded by 76% on the surface, by 67% in the 20-25-cm depth and by 81% in the 45-50 cm depth soil layers. No similar bibliographic data is available for doxycycline, but data referring to other tetracyclines are available. The published measured concentrations are falling into a wide range of 0.004–0.9 mg/kg. Most of the data are not directly comparable because the applied types of antibiotics and the experimental conditions were different in these studies. Data on tetracycline concentrations in deeper soil layers are also not commonly present in publications. According to results of the literature, tetracycline could not be found in detectable amounts at 60 cm under the top soil. The reason why doxycycline could be detected in 25 cm and 50 cm depths in our experiment is due to the effect of ploughing which drives the antibiotic into the deeper layer, otherwise the mobility of tetracyclines in soil is very poor. The calculated half-life for doxycycline in the present study was 66.5 days (9.5 weeks), 76.3 days (10.9weeks), 59.4 days (8.5weeks) at depths of 0-5 cm, 20-25 cm and 45-50 cm, respectively. These values are higher than those reported for other tetracyclines. The differences may be partly due to the phisico-chemical differences between doxycycline and older tetracyclines, and different environmental conditions and also to soil characteristics. The results of our experiment indicate the potential presence of doxycycline in different soil layers 20 weeks after fertilization with composted manure. The detected doxycycline concentrations (0.11 mg/kg and 0.14 mg/kg) in 20-25 cm soil depths 8 weeks after spreading the manure onto the arable land are higher than the criterium (0.1 mg/kg) declared in relevant EMEA (EMA) guidelines, but the concentration (0.06 mg/kg) on the 14th and 20th weeks proved to be lower than the criterium.

The potential effect of doxycycline on soil microorganisms was studied by the nitrogen transformation test. According to the results on day 7, an increase in the quantity of nitrate can be found in each soil sample. The overall increase in the content of nitrate is due to the addition of lucerne meal as an organic substrate and its utilization by soil microorganisms. The fact that the amount of nitrate in soils treated with doxycycline was less after one week incubation period than in the control soil (33-60%) indicates the inhibitory effect of doxycycline on soil microbial activity. After the 14 and 28 days of incubation period, the difference of nitrate production in soil samples seemed to be balanced and appeared to be similar in each soil container, and no significant differences were found in the effect among soils treated with different quantities of doxycycline. This lack of nitrate production difference may be explained by a possible adaptation of the microbial community structure and diversity due to the presence of the antibiotic in the soil. According to literature the antibiotics can exert a temporary selective pressure on soil microorganisms even at environmentally relevant concentrations and they can cause a shift from soil bacteria to soil fungi. Other authors have shown that diversity can attain its original value in a given time after adding antibiotic to soil, whereas changes in the microbial community structure were permanent. In summary, it was found that doxycycline in environmentally relevant concentration considerably inhibited the microbial nitrogen transformation temporarily, but at the end of the experiment on day 28, the rate of inhibition was under the criterium declared in relevant guides.

Since the redox potential of the environment (medium) can be changed by microbial activity (multiplication and/or metabolic rate), in our study the potential effect of antibiotics added to soil samples was examined by measuring the changes of the redox potential. The possible differences according to the type of soils were also investigated. All the three antibiotics exerted inhibitory effect. Doxycycline showed the most marked effect indicated by the increased detection time. The effect of enrofloxacin and lincomycin was the strongest in sand soil and volcanic ash soil. The inhibitory effect of antibiotics could be detected at the concentration of 200 μg/kg. The soil type influenced the effect of antibiotics on soil microflora. Enrofloxacin had no effect in silty clay, loam soil and silt loam soil.

The minimal inhibitory concentration of the three antibiotics was different in every soil types. The minimal inhibitory concentration of doxycycline in volcanic ash soil and silt loam soil proved to be < 20 μg/kg, in loam soil it was more than 20 μg/kg. The minimal inhibitory concentration of enrofloxacin was 30-60 μg/kg in sand soil, silty clay, loam soil and volcanic ash soil. The minimal inhibitory concentration of lincomycin was 15-60 μg/kg. The results of the study indicate that doxycycline and lincomycin and in case of certain soil types also enrofloxacin had effect on redox potential at concentration < 100 μg/kg. Antibiotics may have bactericid or bacteriostatic effect on microorganisms, but in our study the number of viable cells was similar in the control and at the different doxycycline concentrations. It may be explained the presence of resistant bacteria or genes or by the changed microbial community structure and diversity. The considerable change of redox potential accompanied with unchanged number of microorganisms indicate an inhibitory effect on microbial metabolic rate. Since the method used in the study is simple, fast and sensitive, it may be applied in ecotoxicological studies.

New scientific results

1. The degradation half life of doxycycline in composted manure was first determined in field and *in vitro* studies. Also this is the first data on doxycycline concentration in manure after 3 months composting period. 66.9 % and 89.3 % of the initial doxycycline concentration in manure was degraded during 3 months ripening, but still significant amount of doxycycline may enter into the soil.

2. After spreading the manure to arable land, the concentration change and degradation half life of doxycycline was firstly determined in different soil depth as a function of time. Our results indicate that doxycycline can be detected in different soil depths after 20 weeks and both in the superficial layer and in 20-25 cm depth the concentration is over the 100 μg/kg limit.

3. In the literature this was the first time of detecting the effect of doxycycline on nitrogen transformation activity of soil flora. It was found that doxycycline in environmentally relevant concentrations transiently inhibited the nitrogen transformation of microbes but the measure (according to the related guideline) was below of the biologically relevant level.

4. First time was measured the effect of doxycycline and further two antibiotics on redox potential change (relevant indicator of soil microbial activity). It was determined that doxycycline, enrofloxacin and lincomycin in environmentally relevant concentration can increase the detection time indicating inhibitory effect on microbial activity. The intensity of the effect depends on the type of soil.

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